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Enhanced immunogenicity of DNA fusion vaccine encoding secreted hepatitis B surface antigen and chemokine RANTES

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Abstract

To increase the potency of DNA vaccines, we constructed genetic fusion vaccines encoding antigen, secretion signal, and/or chemokine RANTES. The DNA vaccines encoding secreted hepatitis B surface antigen (HBsAg) were constructed by inserting HBsAg gene into an expression vector with an endoplasmic reticulum (ER)-targeting secretory signal sequence. The plasmid encoding secretory HBsAg (pER/HBs) was fused to cDNA of RANTES, generating pER/HBs/R. For comparison, HBsAg genes were cloned into pVAX1 vector with no signal sequence (pHBs), and further linked to the N-terminus of RANTES (pHBs/R). Immunofluorescence study showed the cytoplasmic localization of HBsAg protein expressed from pHBs and pHBs/R, but not from pER/HBs and pER/HBs/R at 48 h after transfection. In mice, RANTES-fused DNA vaccines more effectively elicited the levels of HBsAg-specific IgG antibodies than pHBs. All the DNA vaccines induced higher levels of IgG_{2a} rather than IgG_1 antibodies. Of RANTES-fused vaccines, pER/HBs/R encoding the secreted fusion protein revealed much higher humoral and $CD8^+$ T cell-stimulating responses compared to pHBs/R. These results suggest that the immunogenicity of DNA vaccines could be enhanced by genetic fusion to a secretory signal peptide sequence and RANTES.

Keywords: DNA vaccine; Genetic fusion; Chemokine; RANTES; Secretion signal

Introduction

Despite significant progress made, the immunogenicity of DNA vaccines still must be improved. One of the factors contributing to the low efficacy of intramuscularly administered DNA vaccines could be the expression of DNA vaccines in the muscle cells lacking immune costimulatory molecules such as B7 (McKenzie et al., 2001). Moreover, the cytoplasmic localization of the expressed antigen proteins in the muscle cells could limit the exposure of antigens to the professional immune cells (Boyle et al., 1997). To overcome such problems, it might be desirable to develop new genetic immunization systems that can allow the cellular secretion of the antigen proteins after expression and the targeting of the secreted proteins to the immune cells.

The fusion of immune cell-recruiting agent to an antigen might be one way to deliver the antigen to the immune system. Boyle et al. (1998) reported the enhanced immune responses to a DNA vaccine encoding a fusion antigen that was directed to the sites of immune induction. Recently, immune cell-recruiting chemokines have been studied as molecular adjuvants of DNA vaccines (Eo et al., 2001; Nomura et al., 2001). A previous study demonstrated that genetic fusion of chemokines to a self-tumor antigen could induce protective and T cell-dependent antitumor immunity (Biragyn et al., 1999). Though most of chemokine-fused

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DNA vaccines have focused on the treatment of tumors, a recent report demonstrated the new application of chemokine-fused DNA vaccines for prevention of viral infection (Biragyn et al., 2002). In the study, they reported that the DNA vaccines encoding human immunodeficienty virus-1 glycoprotein 120 fused with monocyte chemoattractant protein-3 or macrophage-derived chemokine could increase the titers of virus-neutralizing activity. However, there is lack of understanding of the effect of endoplasmic reticulum-targeting secretory signal on the immunogenicity of chemokine-fused DNA vaccines.

In this study, we tested whether the humoral and cellular immunogenicity of DNA vaccines could be improved by fusion with a chemokine and, if so, whether the immunogenicity could be further affected by the cellular fate after expression. As a chemokine, RANTES (regulated on activation normal T cell expressed and secreted) has been used in this study. The major receptors of RANTES, CCR1 and CCR5, have been reported to mediate endocytosis (Solari et al., 1997; Mueller et al., 2002), which might be important for intracellular delivery and processing of secreted chemokine-fused antigen. Using hepatitis B surface antigen (HBsAg) as a model antigen, we report here the enhanced humoral immune responses and $CD8^+$ T cell-stimulating effects of HBsAg DNA vaccine encoding an endoplasmic reticulum (ER)-targeting secretion signal peptide sequence and RANTES.

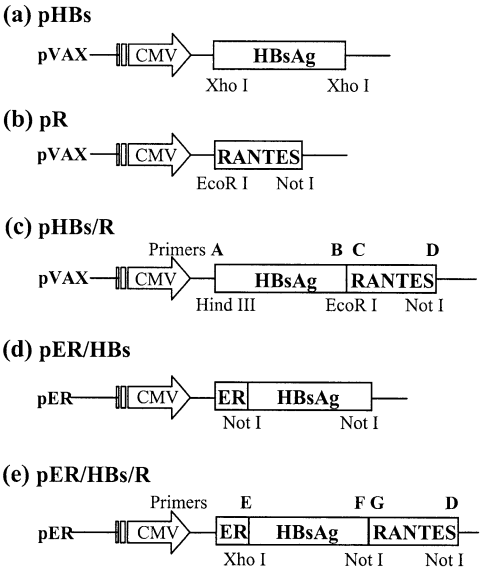

Fig. 1. Construction scheme of HBsAg DNA vaccines. (a) pHBs was generated by cloning HBsAg gene fragment into the *Xho*I site of pVAX1 vector. (b) The cDNA fragment of RANTES was inserted into *Eco*RI/*Not*I sites of pVAX1 vector. (c) HBsAg gene was amplified using primers A and B, digested with *Hin*dIII and *Eco*RI respectively, and inserted into the *Hin*dIII/*Eco*RI sites of pVAX1. The polymerase chain reaction products of RANTES, amplified using primers C and D, were cut with *Eco*RI and *Not*I, respectively, and inserted into the *Eco*RI/*Not*I sites in pVAX1. (d) HBsAg gene fragment was inserted into the *Not*I site pCMV/myc/ER. (e) HBsAg gene was amplified by using primers E and F, digested with *Xho*I and *Not*I, respectively, and inserted into the *Xho*I/*Not*I sites of pVAX1 vector. The cDNA fragment of RANTES was then amplified using primers G and D, digested with *Not*I, and inserted into the *Not*I site of pCMV/myc/ER.

Results

Effect of an ER-targeting signal peptide on the cellular fates of HBsAg

Various HBsAg DNA vaccines were constructed by simple insertion of gene fragments or by insertion of digested polymerase chain reaction (PCR) products after amplification with primers having desired restriction sites (Fig. 1). Table 1 shows the sequences of oligonucleotide primers used for construction of genetic vaccines. pHBs encoding HBsAg lacks ER-targeting secretion signal (Fig. 1a). In contrast, pER/HBs was constructed to design a DNA vaccine encoding secreted HBsAg (Fig. 1d). Two types of HBsAg/RANTES fusion vaccines, p/HBs/R (Fig. 1c) and pER/HBs/R (Fig. 1e), were constructed to encode nonsecretory and secretory form of fusion proteins, respectively.

To test whether the HBsAg genetic fusion constructs could express HBsAg in the cell levels, and if so, whether the mRNA expression levels differ among the various DNA constructs, the expression levels of HBsAg were measured at 6 h after transfection. The presence of mRNA transcripts of HBsAg was observed in all the DNA constructs except the group treated with pR (Fig. 1b) lacking HBsAg moiety (Fig. 2a). Moreover, semiquantitative reverse transcription (RT)-PCR results show that there were no significant differences in the expression levels of HBsAg among the constructs at 6 h after transfection (Fig. 2b).

Though there was no difference in the expression levels of HBsAg at an early time point after transfection, the presence of an ER-targeting signal peptide sequence in the expression vector affected the trafficking of expressed proteins in the cells. The immunofluorescence study measured at 48 h after transfection showed that the cellular fates of expressed proteins contrasted between the DNA vaccines after transient transfection. The cells transfected with pHBs or pHBs/R showed the immunofluorescence of HBsAg proteins in the cytoplasm (Fig. 3a and b), whereas the cells transfected with pER/HBs or pER/HBs/R displayed little cellular immunofluorescence (Fig. 3c and d). Expression of RANTES was observed in the cells transfected with pR and with DNA fusion vaccines encoding RANTES moieties such as pHBs/R and pER/HBs/R (Fig. 4a). However, the secretion of RANTES into cell culture supernatant was shown in the group transfected with pR or pER/HBs/R (Fig. 4b).

To estimate the in vivo chemotactic ability of RANTES derived from DNA constructs such as pR and pER/HBs/R, the expression of chemokine receptor CCR5 was measured following the intramuscular injection of various plasmid DNA. The muscle tissues from the untreated mice showed

Table 1
The sequences of oligonucleotide primers used to construct expression plasmids

Primer	Sequence (5′–3′)	Sense or antisense
A	ACCCAAGCTTGGTACCGGGCC *Hin*dIII (start codon located at 37 bp downstream)	Sense
B	TTAGGAA[TTC]AATGTATACCCA *Eco*RI (stop codon change, TTA → TTC)	Antisense
C	CATGAATTCCCCACAGCGTTTGC *Eco*RI (start codon located at 20 bp downstream)	Sense
D	CAACGCGGCCGCAGCTGACATAC *Not*I (stop codon located at 132 bp downstream)	Antisense
E	CCCCTCGAGGATTGGGGACCCTG *Xho*I (start codon located at 22 bp downstream)	Sense
F	GTTGCGGC[CGC]AATGTATACCCAAAGAC *Not*I (stop codon change, TTA → CGC)	Antisense
G	CCCACAGCGGCCGCCGCGGGTAC *Not*I (start codon located at 15 bp downstream)	Sense

lack of CCR5 expression. pVAX1 expression vector alone induced slight expression of CCR5. In contrast, both pR and pER/HBs/R revealed higher expression of CCR5 in the muscle tissues than did pVAX1 vector (Fig. 5).

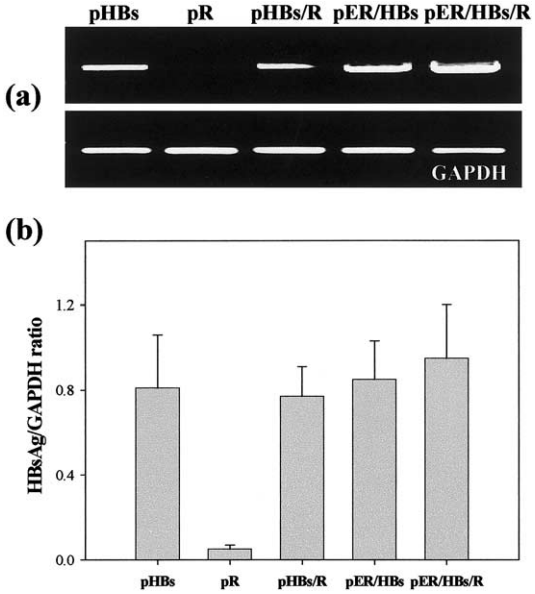

Fig. 2. mRNA expression levels of HBsAg in C2C12 cells. (a) C2C12 cells were transfected with various DNA constructs. At 6 h after transfection, the mRNA expression of HBsAg in the cell pellets was measured by reverse transcription–polymerase chain reaction. The cDNA samples were amplified by using specific primers of HBsAg and GAPDH. (b) The density ratios of HBsAg/GAPDH were determined for each DNA construct. The ratio of HBsAg/GAPDH was obtained by dividing the cDNA band density of HBsAg with that of the corresponding GAPDH.

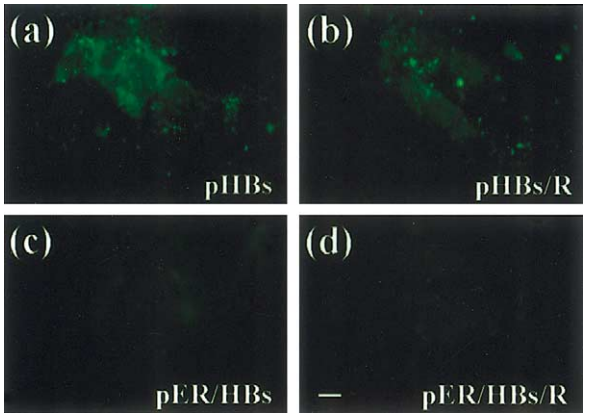

Fig. 3. Immunofluorescence of HBsAg protein in C2C12 cells. C2C12 cells, grown on coverslips, were transfected with pHBs (a), pHBs/R (b), pER/HBs (c), or pER/HBs/R (d) for 2 h. At 48 h after transfection, the cells were fixed, permeabilized, and incubated with anti-HBsAg antibody. The cells were then incubated with fluorescein-conjugated secondary antibody and observed under a fluorescence microscope. The experiment was repeated three times with similar results. Scale bar: 10 μm.

Humoral immune responses to genetic fusion vaccines encoding HBsAg and RANTES

The induction of HBsAg-specific antibodies was affected by the genetic fusion of HBsAg with RANTES and by the cellular fate of expressed proteins. Fig. 6a shows that the serum levels of IgG antibodies were significantly higher in the mice immunized with pER/HBs/R than with pER/HBs,

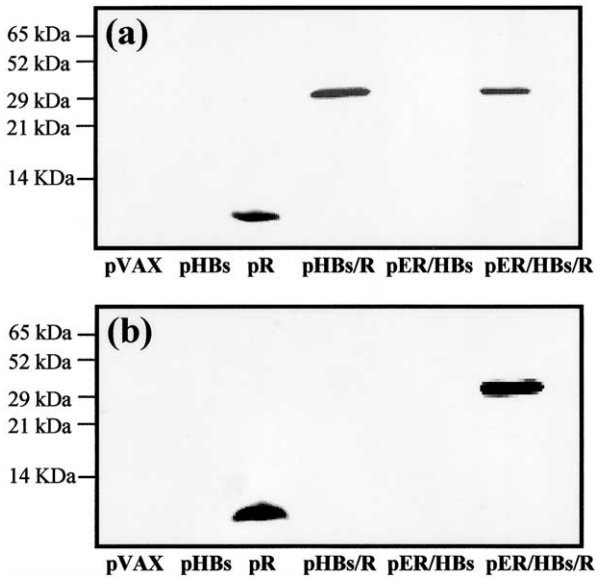

Fig. 4. Western blot of RANTES in C2C12 cells. C2C12 cells were transfected with each plasmid DNA using Lipofectamine. At 24 h after transfection, the cell pellets (a) and supernatants (b) were collected. The presence of RANTES proteins in each fraction was tested by Western blot using a goat anti-RANTES antibody and horseradish peroxidase-conjugated anti-goat IgG antibody. The experiment was repeated three times with similar results.

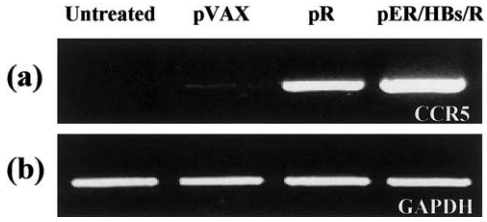

Fig. 5. mRNA expression of CCR5 in muscle tissues. (a) Mice were intramuscularly injected with various DNA constructs. As a control, untreated mice were used. At 48 h after administration, the mRNA expression of CCR5 in the muscle tissues was measured by reverse transcription–polymerase chain reaction. The cDNA samples were amplified by using specific primers of CCR5 (a) and GAPDH (b). The result is the representative figures of three separate experiments.

indicating the adjuvant effect of genetically fused RANTES. Moreover, the higher levels of IgG were observed in pER/HBs encoding secretory HBsAg compared with pHBs lacking the ER-targeting secretory signal peptide sequence. Of RANTES-fusion vaccines, the induction of IgG responses was higher in pER/HBs/R compared to pHBs/R (Fig. 6b). Moreover, the DNA vaccines expressing the secreted HBsAg protein fused to RANTES differed from the physical mixture of DNA vaccine encoding secreted HBsAg and the plasmid encoding RANTES. The coadministration of pER/HBs and pR revealed IgG levels lower than did pER/HBs/R (Fig. 6b).

All the genetic vaccines tested in this study induced the higher levels of IgG_{2a} than IgG_1 antibodies. The HBsAg genetic vaccine with an ER-targeting secretion signal sequence, pER/HBs, produced the higher levels of IgG_{2a} and IgG_1 relative to the DNA vaccine encoding cytoplasmic HBsAg, pHBs (Fig. 7). Among DNA vaccines tested, pER/HBs/R showed the highest IgG_1 and IgG_{2a} levels (Fig. 7).

$CD8^+$ T cell-stimulating effects of HBsAg genetic fusion vaccines

Similar to the humoral immune responses, the $CD8^+$ T cell-stimulating effects of HBsAg DNA vaccines were increased by genetic fusion to RANTES and by introduction of a secretor signal sequence. The DNA vaccines expressing HBsAg fused to RANTES (pHBs/R and pER/HBs/R) displayed the significantly higher $CD8^+$ T cell-stimulating activity relative to pHBs encoding HBsAg alone (Fig. 8).

The DNA vaccines expressing the secretory HBsAg showed the higher $CD8^+$ T cell-stimulating effects than did other vaccines encoding cytoplasmic HBsAg. Between the DNA vaccines encoding HBsAg alone, pER/HBs showed the higher $CD8^+$ T cell-stimulating activity than did pHBs. Of RANTES-fusion vaccines, pER/HBs/R encoding secreted form of HBsAg/RANTES fusion protein showed the higher $CD8^+$ T cell-stimulating activity in comparison with pHBs/R encoding cytoplasmic form of HBsAg/RANTES fusion protein. The administration of pHBs/R and the coadministration of pER/HBs and pR did not significantly differ in the cellular immune responses. However, the coadministration of pER/HBs and pR displayed significantly lower effects relative to pER/HBs/R.

Discussion

In this study, we demonstrated that the genetic fusion with chemokine RANTES could enhance the immunogenicity of HBsAg DNA vaccines. Moreover, our results indicate the importance of cellular fate in the immunogenicity of genetic fusion vaccines.

The immunofluorescence data indicate that the cellular fates of DNA vaccines could be altered by the presence of an ER-targeting secretion signal peptide sequence. We ob-

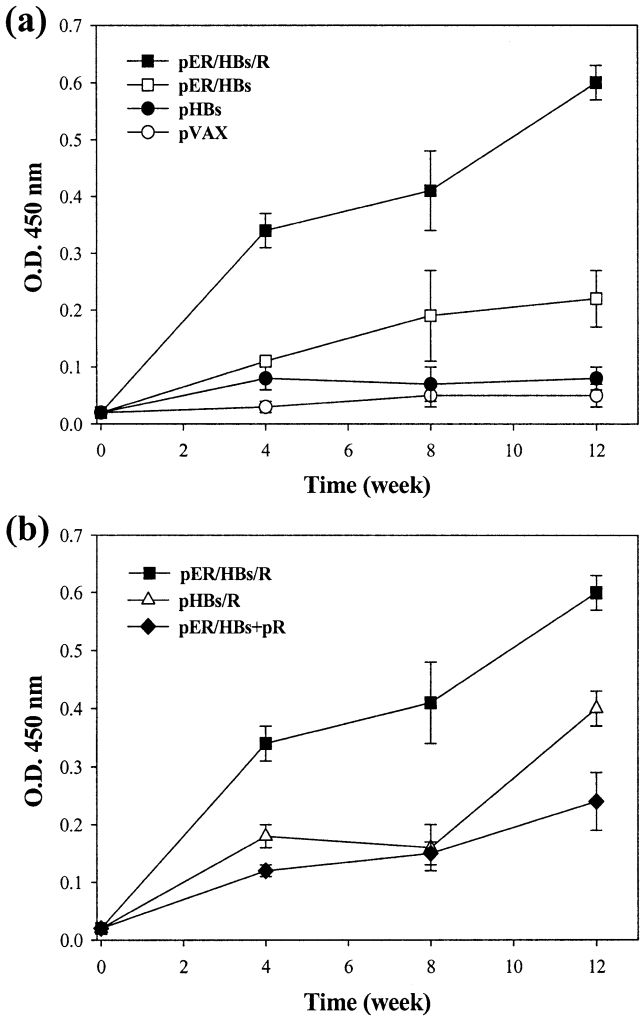

Fig. 6. IgG antibody responses of DNA vaccines. Mice were immunized by intramuscular injection of 100 μg of each plasmid DNA vaccine at 0, 4, and 8 weeks. At various time points after the first immunization, the blood was collected from the tail vein, and the serum levels of anti-HBsAg IgG antibodies were determined by enzyme-linked immunosorbent assay. The results are expressed as mean ± SE values ($n = 5$).

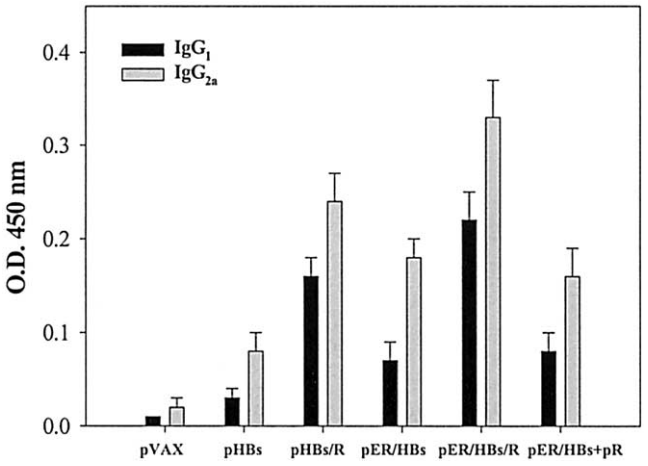

Fig. 7. IgG_1 and IgG_{2a} subtype antibody responses of DNA vaccines. Mice were immunized by intramuscular injection of 100 μg of each plasmid DNA vaccine at 0, 4, and 8 weeks. At various time points after the first immunization, the blood was collected from the tail vein, and the serum levels of anti-HBsAg IgG_1 and IgG_{2a} subtype antibodies were determined by enzyme-linked immunosorbent assay. The results are expressed as mean $\pm$ SE values ($n = 5$).

served the lack of immunofluorescence observed at 48-h transfection with pER/HBs or pER/HBs/R (Fig. 3). Given the similar mRNA expression levels of HBsAg derived from various DNA constructs in the cell pellets at 6 h after transfection (Fig. 2), it is unlikely that pER/HBs and pER/HBs/R could be less efficiently transcribed to HBsAg than pHBs. Rather, the lack of immunofluorescence appears to be due to the secretion of expressed HBsAg/RANTES fusion proteins to the extracellular environments, supporting the strength of the artificial ER-targeting signal sequence in modulating the cellular fate of HBsAg and RANTES fusion proteins.

In our Western blot study (Fig. 4), pHBs/R did not secrete RANTES into supernatant at the time point tested. pHBs/R was constructed to encode RANTES containing its endogenous secretory signal sequence following the gene of HBsAg. It appears that the fusion of RANTES secretory signal to the N-terminal of HBsAg might have resulted in the protein systhesis in cytosolic free ribosomes, rather than ER-bound ribosomes leading to the secretion of expressed proteins. Currently, it remains unclear by which mechanisms pHBs/R construct with a secretory signal in the middle of the antigen and RANTES sequence resulted in the cytosolic protein synthesis. However, the possibility exists that the binding of a secretory signal sequence with a signal receptor might not be optimal in the HBsAg/RANTES chimeric fusion gene structure.

Expression of CCR5 has been studied as one of the ways to measure the biological effects of RANTES. CCR5, one of RANTES receptors, has been known to be highly expressed on monocytes and dendritic cells showing chemotaxis to RANTES. The cells showing the high grade chemotaxis to RANTES were shown to express the higher levels of CCR5 receptors (Cockwell et al., 2002). If monocytes and dendritic cells migrated in response to RANTES expressed from the DNA constructs, the induced migration of those immune cells may increase the levels of CCR5 in the injection sites. Indeed, to characterize the biological function of synthetic RANTES, Vita et al. (2002) showed that biotinylated RANTES could modulate the expression of CCR5 expression in the same manner as unmodified RANTES. Based on these findings, our observation that both pR and pER/HBs/R showed the higher expression levels of CCR5 than did pVAX1 vector at the injection site (Fig. 5) may suggest that HBs/R fusion protein expressed from pER/HBs/R could retain the chemotactic ability of RANTES.

The humoral immunogenicity of HBsAg DNA vaccines was enhanced by genetic fusion with RANTES. The stronger IgG levels of pER/HBs/R in comparison with pER/HBs imply that the genetic fusion of HBsAg to RANTES might be more effective in inducing humoral immune responses compared to HBsAg alone. Previously, the increased humoral antibody responses were observed after treatment with RANTES (Lillard et al., 2001). The report suggested that RANTES might enhance the humoral antibody responses through the induction of cytokine and costimulatory molecules and through the expression of cytokine receptors on T lymphocytes.

In addition to genetic fusion with RANTES chemokine genes, the cellular fate appears to be a role as an important factor affecting the immunogenicity of genetic vaccines. Although both pHBs/R and pER/HBs/R encode RANTES-fused HBsAg, the higher humoral responses of pER/HBs/R over pHBs/R indicate that the presence of an ER-targeting secretion signal sequence might be required for higher potency of DNA fusion vaccines. Similarly, the higher im-

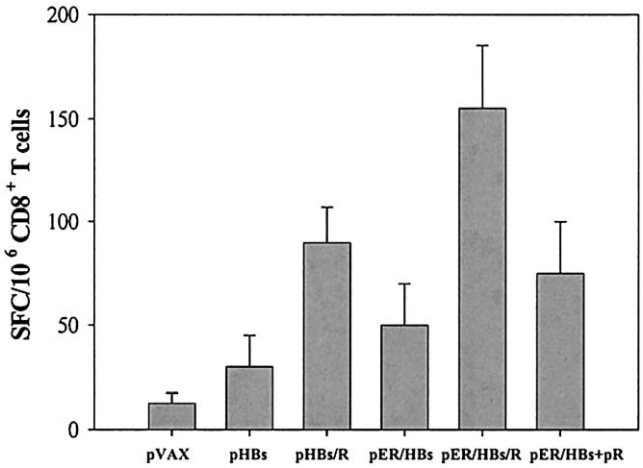

Fig. 8. HBsAg-specific $CD8^+$ T-cell stimulation of DNA vaccines. Mice were immunized by intramuscular injection of 100 μg of each plasmid DNA vaccines at 0, 4, and 8 weeks. At 12 weeks after immunization of mice with various DNA vaccines, mice were killed and $CD8^+$ T cells were isolated. Responses are shown as the number of HBsAg-specific interferon-γ spot-forming cells (SFC)/10^6 $CD8^+$ T cells. The results are expressed as mean $\pm$ SE values ($n = 5$).

mune response of pER/HBs encoding secreted HBsAg in comparison with pHBs indicates the importance of cellular localization in the potency of DNA vaccines. Consistent with our observation, Boyle et al. (1997) reported that cytotoxic T lymphocyte and antibody responses were suboptimal when the cellular location of antigen was cytoplasmic after intramuscular DNA immunization. Improvement of humoral and cellular immune responses was reported when the plasmid DNA encoding the outer surface protein C of *Borrelia burgdorferi* was fused to the native ER-targeting secretory signal sequences of tissue plasminogen activator (Weiss et al., 2000).

The highest IgG induction was observed in the groups immunized with pER/HBs/R. RANTES has been reported to be endocytosed via receptors on dendritic cells and monocytes (Solari et al., 1997). It is speculated that the secreted form of HBsAg and RANTES fusion proteins might be taken up by antigen-presenting cells through chemokine receptors such as CCR1 and CCR5. Moreover, given the chemokine cascade-inducing activity of RANTES in dendritic cells (Fischer et al., 2001), we cannot exclude the possibility that RANTES-mediated chemokine amplification in dendritic cells may also shape the microenvironment, potentially enhancing the immune responses to RANTES-fused HBsAg.

We measured antigen-specific $CD8^+$ T-cell stimulation to test the cell-mediated immunogenicity of various DNA vaccines. Antigen-specific $CD8^+$ T cells are known to be a major host defense against viral infection (Yewdell and Hill, 2002). $CD8^+$ T cells are responsible for the antigen-specific recognition and elimination of virus-infected cells through direct killing and production of antiviral cytokines (Appay and Rowland-Jones, 2002). Several studies have employed antigen-specific $CD8^+$ T-cell responses to test the cell-mediated immunogenicity of DNA vaccines. $CD8^+$ T-cell responses were measured following coimmunization with plasmids expressing different antigens of murine cytomegalovirus (Ye et al., 2002). Antigen-specific $CD8^+$ T-cell responses were studied after immunization of rhesus macaques with human immunodeficiency virus gag DNA vaccines (Caulfield et al., 2002). Given the importance of antigen-specific $CD8^+$ T cells in cell-mediated immunity, we determined the stimulation of HBsAg-specific $CD8^+$ T cells using ELISPOT assay.

The genetic fusion with RANTES chemokine also increased the antigen-specific $CD8^+$ T cell-stimulating effects of HBsAg DNA vaccines. The higher number of HBsAg-specific $CD8^+$ T cells in pER/HBs/R over pER/HBs supports the adjuvant role of RANTES in the induction of cellular immune responses. Previously, the induction of cell-mediated antitumor immunity was reported for lymphoma-specific single chain antigen genetically fused to chemokines such as monocyte chemotactic protein-3 or interferon inducible protein-10 (Biragyn et al., 1999). DNA vaccines encoding RANTES were also shown to enhance $CD4^+$ T cell-mediated protective immunity against herpes simplex virus type 2 (Sin et al., 2000).

In conclusion, our results suggest that the genetic fusion of secreted antigens to RANTES might be a useful approach to enhance the immunogenicity of DNA vaccines.

Materials and methods

Construction of genetic fusion vaccines

The HBsAg-encoding sequence, subtype ayw, was obtained from the plasmid pRc/CMV-HBs(S) (a kind gift of Aldevron, Fargo, ND, USA). The coding sequence of HBsAg was inserted into the *Xho*I site of the pVAX1 expression vector (Invitrogen, CA, USA) generating the plasmid pHBs (Fig. 1a). The eukaryotic expression vector pVAX1 contains the cytomegalovirus early promoter/enhancer sequence and kanamycin resistance gene. Murine RANTES cDNA was kindly provided by Dr. T. Yoshimura (National Cancer Institute, MD, USA). For expression plasmid of RANTES, the cDNA fragment including the native 23-amino acid leader sequence (279-bp) was inserted into *Eco*RI/*Not*I sites of pVAX1 vector, resulting in pR (Fig. 1b). To construct pHBs/R, HBs gene was amplified from pHBs using primers A and B, digested with *Hin*dIII and *Eco*RI, respectively, and inserted into the multicloning sites of pVAX1 vector. Then, RANTES cDNA, amplified from pBluescript/RANTES using primers C and D, was cut with *Eco*RI and *Not*I and inserted into the *Eco*RI and *Not*I sites of pVAX1 vector (Fig. 1c).

To induce the secretion of expressed HBsAg protein from the cells, HBsAg gene was inserted into the multicloning sites of pCMV/myc/ER (Invitrogen, CA, USA) with the cytomegalovirus early promoter/enhancer sequence and an ER-targeting signal sequence in the backbone (Fig. 1d). For pER/HBs, HBsAg-encoding gene fragment was cut from pHBs using *Not*I, then inserted into the *Not*I site of pCMV/myc/ER vector. For genetic fusion of RANTES to HBsAg (Fig. 1e), HBsAg-encoding fragment was amplified from pHBs using primers E and F, cut with *Xho*I and *Not*I, then inserted into *Xho*I and *Not*I sites of the secretion signal pCMV/myc/ER vector. Then, RANTES cDNA was amplified using primers G and D, digested with *Not*I, and inserted into the *Not*I site of pCMV/myc/ER vector, generating pER/HBs/R. All the plasmid DNAs in this study were amplified using *Escherichia coli* Top10F and purified using Qiagen Giga Prep Kit (Qiagen, CA, USA).

Semiquantitative analysis of HBsAg expression levels in C2C12 cells

To determine the mRNA expression levels of HBsAg genes, semiquantitative RT-PCR was performed. C2C12 mouse muscle cells (kindly provided from Dr. YI Yeom, Korea Research Institute of Bioscience and Biotechnology,

Korea) were grown on a 12-mm coverslip at a density of 1×10^5 cells and transfected with pHBs, pR, pHBs/R, pER/HBs, or pER/HBs/R using Lipofectamine (GibcoBRL, MA, USA) for 2 h. After 6-h incubation, total cellular RNA was extracted from the cells using TRIzol reagent (GibcoBRL). One microgram of total RNA was reverse transcribed, and amplified by PCR using primers as described previously (Lai et al., 1996). The murine housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The same amount of cDNA was amplified by PCR for GAPDH using the primers as described previously (Oh et al., 2001). The PCR products were electrophoresed on a 2% agarose gel, and visualized with ethidium bromide. The density of each band was measured using a gel-doc image analyzer (Vilber Lourmat, France). The band intensities of HBsAg were normalized with those of corresponding GAPDH.

Immunofluorescence

The cellular fate of HBsAg was tested by immunofluorescence. C2C12 mouse muscle cells were grown on a 12-mm coverslip at a density of 1×10^5 cells and transfected with pHBs, pR, pHBs/R, pER/HBs, or pER/HBs/R using Lipofectamine (GibcoBRL) for 2 h. At 48 h after transfection, the cells were fixed at room temperature for 20 min using a modified paraformaldegyde-lysine-periodate fixative (Oh and Swanson, 1996). Next, the cells were washed, permeabilized for 20 s using cold methanol, and incubated for 2 h with mouse anti-HBsAg monoclonal antibody (1:250, Aldevron, USA). The cells were then washed and incubated for 1 h with fluorescein-conjugated goat anti-mouse IgG antibody (1:250, Pierce, IL, USA). Finally, the cells were washed and mounted in a phostostabilizer (90% glycerol, 10% phosphate buffer, 1 mg/ml phenylenediamine), and observed under a fluorescence microscope (Nikon H-III, Japan).

Western blot analysis

The expression of RANTES in the series of DNA vaccine constructs was tested by using Western blot. C2C12 cells (1×10^6 cells) were seeded onto 60-mm tissue culture plates and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum until the cells reached approximately 80% confluence. The cells were transfected with plasmid DNA using Lipofectamine as specified by the manufacturer. At 24 h after transfection, the cell pellets and supernatants were collected and stored at −70°C until analysis. For Western blot analysis, the nitrocellulose blot membranes were incubated with goat anti-murine RANTES antibody (1:2000, Santacruz, CA, USA). For detection of RANTES, horseradish peroxidase-conjugated anti-goat IgG antibody (1:2000, Pierce) was used as the secondary antibody.

CCR5 expression in muscle tissues

The expression of CC chemokine receptor, CCR5, was measured in the muscle tissues using RT-PCR. Mice were intramuscularly administered with pVAX1, pR, or pER/HBs/R at a dose of 100 μg of plasmid DNA in 20 μl of phosphate-buffered saline. At 48 h after injection, the muscle tissues near the injection sites were removed and total RNA was extracted from the tissues using TRIzol reagent (GibcoBRL). One microgram of total RNA was reverse transcribed, and amplified by PCR using primers specific for murine CCR5 receptors as described previously (Fischer et al., 2000). The same amount of cDNA was amplified by PCR for GAPDH as described previously (Oh et al., 2001).

Immunization of mice

For immunization, female BALB/c mice were supplied from Seoul National University animal center (Seoul, Korea). The mice were used at 6–8 weeks of age and received food and water ad libitum. Each mouse was given intramuscular injections into the left quadriceps at a dose of 100 μg of plasmid DNA in 20 μl of phosphate-buffered saline. All mice were boosted with equal doses after 4 and 8 weeks from the first injection.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to analyze the induction of humoral immune responses against HBsAg in the immunized mice. Serum samples were collected by tail bleeding at different time points after immunization and analyzed for the presence of HBsAg-specific antibodies. A 96-well plate was coated with recombinant HBsAg proteins (2 μg/ml, kindly provided from LG Chem., South Korea). After blocking of the plates with 2% bovine serum albumin (BSA), dilutions of test sera (1:100) were added to each well and incubated for 2 h at room temperature. The plates were then washed, and the levels of bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG_1, or IgG_{2a} antibodies. Color was generated by adding 1-Step Turbo TMB-ELISA (Pierce), and absorbance at 450 nm was measured by using an ELx-800 ELISA reader (Bio-Tek Instruments, VT, USA).

Enzyme-linked spot (ELISPOT) assay

At 12 weeks after the first immunization of mice with various DNA vaccines, mice were killed and the spleens were removed. The spleen cells were then mixed with magnetic beads coated with anti-mouse CD8 antibody (Macs, Germany), and $CD8^+$ T cells were isolated according to instructions of the manufacturer. Multiscreen 96-well plates, MAIP NOB 10 (Millipore, MA, USA), were coated with a monoclonal antibody against human interferon-gamma (IFN-γ), clone R4-6A2 (Pharmingen, CA, USA).

The following day, $CD8^+$ T cells were plated at 2×10^5 cells per well in 200 μl of complete RPMI medium (50 U/ml penicillin and 50 μg/ml streptomycin). HBsAg-specific CD8 epitope peptides (IPOSLDSWWTSL, Schirmbeck et al., 1998) were supplied from Peptron Inc. (Daejeon, South Korea), and added to the $CD8^+$ T-cell cultures at a final concentration of 2 μg/ml. As a positive control, concanavalin A (Sigma, MO, USA) was added to control wells at a final concentration of 2 μg/ml. The plates were incubated at 37°C, 5% CO_2, for 36 h. After washing the plates, biotinylated anti-mouse IFN-γ, clone XMG1.2 (Pharmingen) was added at a concentration of 2 μg/ml and the plates were incubated at room temperature for 2 h. After three washings, streptavidin-conjugated alkaline phosphatase (Vector, CA, USA) was added at 1:2000 dilutions. After incubation at room temperature for 1 h, the plates were washed, and the spots were developed with 1-Step NBT/BCIP reagent (Pierce). The number of spot-forming cells was counted by using an image analyzer (Vilber Lourmat, France).

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